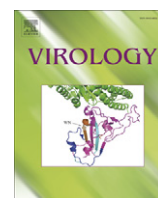


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Infection of bovine dendritic cells by rinderpest or measles viruses induces different changes in host transcription

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ABSTRACT

The morbilliviruses are a closely related genus which are very similar in their sequences and share a common receptor, but nevertheless show significant restriction in the host species in which they cause disease. One contribution to this restriction might be the nature of the hosts' responses to infection. We have used microarrays to study the changes in the transcriptome of bovine dendritic cells after infection with wild-type (pathogenic) and vaccine (apathogenic) strains of rinderpest virus (RPV), a bovine pathogen, and a wild-type isolate of measles virus (MV), a morbillivirus that causes disease only in humans and some other primates. We found that, as previously observed in human cells, MV induces a rapid interferon response, while that induced by RPV was delayed and much reduced in magnitude. Pathogenic and apathogenic RPV also showed significant differences, with the latter inducing a slightly higher interferon response as well as significant effects on transcription of genes involved in cell cycle regulation.

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Introduction

The paramyxoviruses are a large group of viruses causing diseases of many different species. The individual viruses normally only cause disease in a limited range of hosts, although there are exceptions, such as *Canine distemper virus* (CDV), which has a host range encompassing many types of carnivorous mammal, including seals, lions and ferrets, as well as domestic and wild dogs. More commonly, closely related viruses exist which are each specific to a particular host, e.g. bovine and human respiratory syncytial viruses. Our laboratory has been actively investigating the determinants of pathogenicity of the paramyxovirus *Rinderpest virus* (RPV), a member of the genus *Morbillivirus* and the causative agent of a highly contagious and often fatal disease of cattle. RPV is also the most closely related virus to the human pathogen *Measles virus* (MV). Indeed, it has been suggested that MV came into human populations from RPV (Norrbj et al., 1992); it is assumed that an ancestral cattle morbillivirus passed to a human host in which it could replicate inefficiently (but not necessarily

apathogenically), and adapted to that host over time. The possibility of transfer from one type of host to another is particularly important given that both MV and RPV are the target of world-wide eradication campaigns, which will lead to the relevant host populations eventually having little or no acquired immunity to this group of viruses, while other related viruses (e.g. CDV, *Phocid distemper virus* (PDV), and *Peste des petits ruminants virus* (PPRV)) remain in circulation. It is therefore important that a thorough understanding is acquired of the interactions of these viruses with their respective hosts and the underlying mechanisms of host-specificity of disease.

Although the morbilliviruses are restricted in the host range in which they cause disease, they all seem able to infect most mammalian species subclinically, at least enough to cause seroconversion. These observations *in vivo* were explained in part by the discovery that all morbilliviruses tested appear to use the highly conserved protein CD150 (also known as signalling lymphocyte activation molecule (SLAM)) as a primary receptor (Baron, 2005; Seki et al., 2003; Tatsuo et al., 2001). This appears to give these viruses a ready ability to enter and adapt to new hosts. In addition to the example of RPV and MV already cited, sequence analysis has shown that PDV was derived from CDV, or vice versa (Kovamees et al., 1991). Forced passage of RPV in rabbits in an attempt to make an attenuated virus for use as a vaccine resulted in a virus that was indeed avirulent in cattle, but was now highly pathogenic in rabbits (Yamanouchi et al., 1974). It is possible that, once MV and RPV have been eradicated and immunisation stopped, another morbillivirus

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may exploit the newly available set of hosts, giving rise to new diseases, possibly severe.

Since individual morbilliviruses can infect many species, but cause disease in few, the specific internal milieu of the infected host cell and the response to viral entry may be critical in determining the outcome of infection. The failure of MV to cause disease in cattle (and the failure of RPV to cause disease in primates) may be determined by differences in the ability of the viruses to use host cell components, or differences in the host responses to the individual viruses. For example, we have found that RPV can block both the induction (Boxer et al., 2009) and the action (Nanda and Baron, 2006) of interferons, and studies on a number of viruses have shown that the ability to block innate immune responses can be a critical factor in determining the pathogenicity of a virus (Bartlett et al., 2008; Devaux et al., 2008; Huang et al., 2003; Parisien et al., 2002). If the abilities of RPV and MV to control innate immune responses are host specific, or otherwise different, this would affect host-specificity of pathogenesis.

We have determined the effects of RPV infection on bovine target cells at the transcriptome level. In order to study whether host-specificity of response to a virus is linked to host-specificity of disease, we have compared not only host cell responses to infection with virulent and avirulent RPV but also responses to virulent MV. The results of these studies, coupled with previously published studies on MV infection of human cells (Sato et al., 2008; Zilliox et al., 2006), suggest that RPV and MV have fundamentally different ways of combating host innate immune responses.

Results

Viruses and target cells

The target cell for these studies had to be a bovine primary (non-transformed) cell (since transformation often alters cell responses to different stimuli) and one that was normally infected by RPV in the host animal. We examined newborn calf oesopharyngeal cells (KOP-R cell line) and primary monocyte-derived dendritic cells (moDCs), since RPV and MV infect respiratory epithelial cells *in vivo*, while B cells, T cells and DCs are major targets of RPV and MV in natural infections. Both cell types could be infected by RPV or MV at >80% when exposed to virus at a multiplicity of infection of 9–10, as assessed by immunofluorescence labelling of cells 16 h post infection (data not shown). Immature DCs (including moDCs) are normally considered as only expressing low levels of the morbillivirus receptor (CD150/SLAM) (Murabayashi et al., 2002). In the absence of an antibody recognising bovine CD150, we were unable to determine the corresponding level of protein expression, but quantitative RT-PCR showed that moDCs had a detectable level of CD150 mRNA, albeit 10-fold lower than in activated cells (data not shown). CD150 mRNA levels in the KOP-R epithelial cells were 600-fold lower than in activated macrophages, and entry into these cells was probably through an alternate receptor (Takeuchi et al., 2003). Since the moDCs could be obtained from multiple animals, they provided us with a way of introducing biological replication into the experiment, and these cells were therefore chosen for the target cells.

moDCs from six individual outbred cattle were infected with virus (or treated with a similar volume of control preparation from B95a cells). At 2, 4, 8 and 16 h post infection, cells were harvested and total RNA prepared as described in Materials and methods. Poly(A)⁺ RNA was amplified, labelled and hybridised to two kinds of arrays, a bovine leukocyte cDNA array (BOTL5) and a bovine long oligo array (BLO) (CAFG, Michigan). Although the results we obtained from the BOTL5 array mirrored those obtained from the BLO array, the variability of the data from the former was much higher, and far fewer genes were identified as differentially expressed. Most of the informative results described below came from the BLO array data.

Overall effects of infection

The LIMMA package can be thought of as performing a gene-by-gene ANOVA (analysis of variance). In order to graphically represent this analysis of variance we used a data reduction technique (principal component analysis (PCA)) which mathematically projects complex multi-dimensional data (in this case the expression of >8000 genes in each data set) onto two dimensions (Misra et al., 2002). In PCA the dimensionality of the data set is reduced by replacing the original variables (individual gene expression values) by a smaller number of newly formed variables that are linear combinations of the original variables while retaining the majority of the information about (variation between) the data sets. The absolute values in the plot have no direct physical correlates, rather they are a descriptive tool to capture the relative degree of difference or similarity between the data sets. While it is necessary to rely on the ANOVA analysis to identify statistically significant differences, the PCA gives a helpful visual representation of the overall effects of infection on the molecular state of host cells.

In the plot of the results of the PCA (Fig. 1), the first principal component (PC) (PC1, x-axis), representing the major amount (66%) of the total variation between the data sets, appears to represent a component of the transcriptional response over time which is common to all experimental groups including the mock-infected, since early time points are at one end of the axis and later time points at the other. The second PC (PC2, y-axis), capturing 13% of the total variation, appears to represent virus-induced molecular changes. We observed that mock-infected cells followed a trajectory characterized by a sharp transition along PC1 between an early (2–4 hpi) and a late (8–16 hpi) infection state. The trajectory followed by cells infected by the pathogenic strain of RPV (RPV-S) is shifted across the second PC relative to that of the mock-infected cells, although overall they are very similar to each other and, until the 16 hpi point, to cells infected by the RPV vaccine strain (RPV-R). Cells infected with MV, on the other hand, are characterized by an early transition across the second PC which suggests a considerable response of the host to MV infection.

Our initial LIMMA analysis was aimed at identifying the different factors that contributed significantly to the overall variance of the data so that all those factors should be included in the ANOVA. We found that there were significant variations in the gene-specific response in different animals, so that there were statistically significant differences in the expression of specific genes between different animals, regardless of the treatment conditions (e.g. see Table II in Supplemental Materials for examples). This was not unexpected, given the

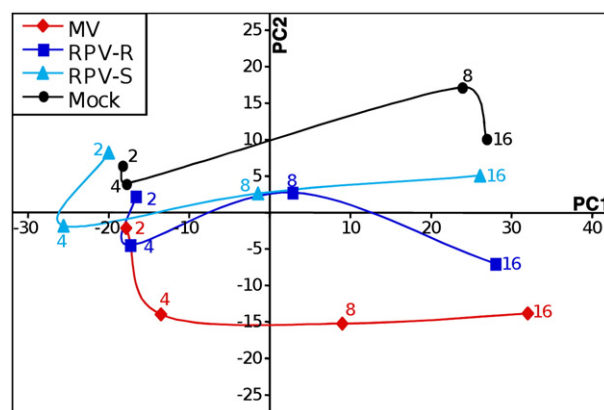


Fig. 1. Principal component analysis of data from the microarray study. The \log_2 of the average expression values (taken over all animals) for each gene in samples from mock-infected cells or cells infected with MV, RPV-R (vaccine strain) or RPV-S (virulent strain) were used for the analysis and the values of the first two components (PC1 and PC2) of the principal component analysis plotted. Numbers next to data points indicate the hours post infection.

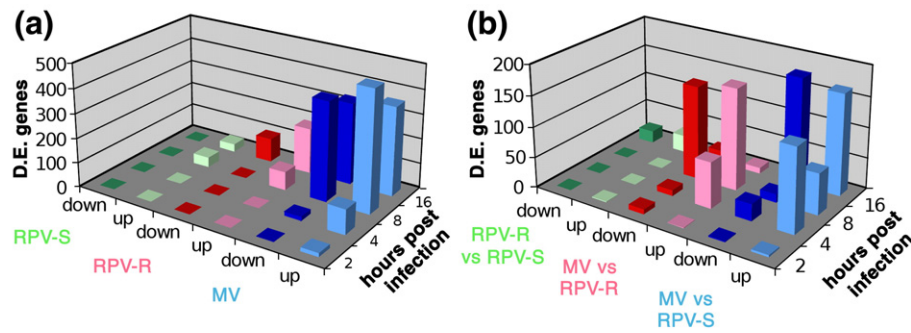


Fig. 2. Numbers of differentially expressed transcripts. Numbers of upregulated or downregulated transcripts identified by linear model analysis at each time point for each virus (a) relative to mock infection and (b) relative to infection with other viruses. In each graph, the dark colours are the numbers of downregulated genes while the light colours are the numbers of upregulated genes. “D.E. genes” = number of genes differentially expressed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

outbred nature of the source animals, and confirmed the utility of working with multiple biological replicates. It was therefore necessary to include animal as a factor in the ANOVA rather than simply average over all biological replicates. We also found clear gene-by-dye effects, as reported by others for two-channel data (Kelley et al., 2008; Liang et al., 2003; Martin-Magniette et al., 2005) (see Table III in Supplemental Materials). Our analysis therefore included treatment (virus and time), animal and dye as factors, and these were the coefficients of the linear model fitted by LIMMA. This model was then used to determine the effects of specific virus/infection time on individual genes (see Materials and methods). We compared virus-infected cells at each time relative to mock-infected at the same times (i.e. MV_2hpi vs Mock_2hpi, MV_4hpi vs Mock_4hpi, etc.). The

immediate results from this analysis were that the MV-infected cells showed a much earlier and stronger response at the transcriptome level than cells infected with RPV (Fig. 2a). Significant changes in transcription of several genes were seen in MV-infected cells at the earliest time point (2 hpi), with more than a hundred genes showing up/downregulation by 4 hpi. In contrast, no significant change to the level of any individual transcript was seen in RPV-infected cells until 8 hpi (Fig. 2a). RPV-R (vaccine strain) infection induced stronger/earlier responses than RPV-S infection, but in neither case was there any detectable change in transcription until 8 hpi. Cluster analysis of the patterns of gene expression in virus-infected cells relative to mock-infected showed similar results to that obtained by PCA on the full data, in that the MV-infected cells clustered separately from the

(a) MV 4hpi

(b) RPV-R 8hpi

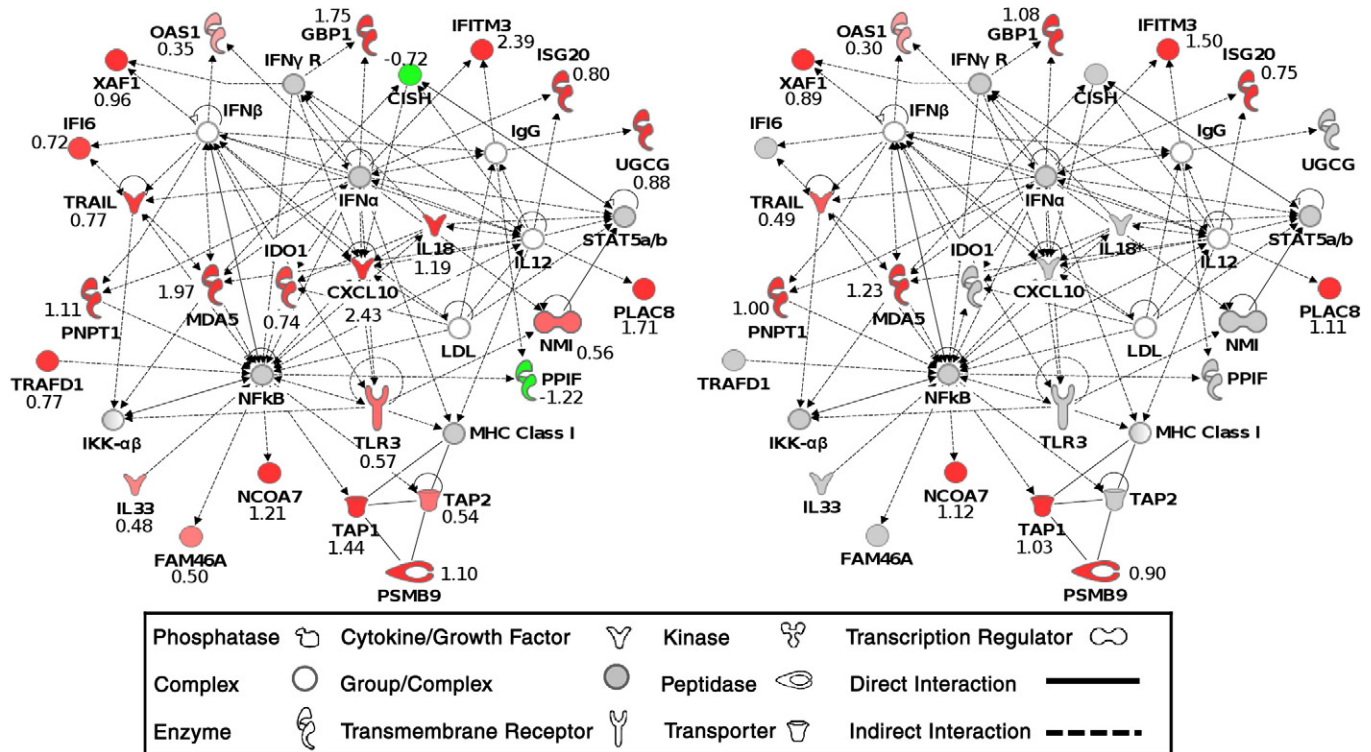


Fig. 3. Comparison of the innate immune response to MV or RPV infection. (a) Interaction network (generated by IPA) of innate immune-related genes based on gene transcripts upregulated (red) or downregulated (green) in MV-infected cells at 4 hpi. The estimated log₂(fold change in expression level) relative to mock-infected cells is shown for each gene having a statistically significant change in expression. (b) The same network overlaid with the data for RPV-R-infected cells at 8 hpi. Cells infected with RPV-S showed a similar pattern except that no significant change was identified for NCOA7 while CXCL10 and NMI were upregulated. Genes are displayed using various shapes that represent the IPA-defined functional class of the gene product, as indicated in the legend.

RPV-infected cells, while the RPV-R and RPV-S data tended to cluster in pairs of the same time point, with the largest difference between RPV-S and RPV-R at 16 hpi (Fig. S2 in Supplemental Materials). We also compared the host response to each virus relative to the other viruses (MV_2hpi vs RPV-R_2hpi, etc.; MV_2hpi vs RPV-S_2hpi, etc.; RPV-R_2hpi vs RPV-S_2hpi, etc.) again showed the major differences as being between MV and RPV (Fig. 2b). Differences between MV and RPV-R were smaller at later times as the RPV-R-infected cells began to respond. The complete sets of analysed data (virus vs mock and virus vs virus) are available as Supplemental Materials.

Array data validation

To validate the results of the statistical analysis of the microarray data, we selected a number of transcripts either identified as significantly up- or downregulated by LIMMA or which are regarded as “housekeeping” genes (normally invariant) and created real-time PCR pairs for the relevant bovine mRNA. Comparison of the relative expression in the virus-infected samples as compared to the mock-infected, as determined by LIMMA, with the difference in Ct (mock minus virus) seen in real-time PCR showed a reasonably good fit for all genes tested (Fig. S3 in Supplemental Materials), in that all treatment-time points identified as showing significant up- or downregulation by LIMMA showed the same change by real-time PCR. The absolute fold changes determined by microarray analysis were smaller than those determined by real-time PCR, as previously reported by others (Wang et al., 2006; Yuen et al., 2002), and this led to some smaller changes in transcription not being identified by the microarray analysis, but the direction and timing of the effects were always correlated.

Effects of infection on transcription of specific genes

The lists of genes up- and downregulated by each virus at different times, and the size and direction of change in expression, as

Table 1

Transcriptional responses of IFN-responsive genes and genes associated with DC activation and innate immune responses.

Gene name	MV	RPV-R	RPV-S
BOLA-DMB	1.1501327	NS	NS
BOLA-DQB	0.3271499	NS	NS
CARD11	0.22852	NS	NS
CCL3	1.2361403	NS	NS
CD200	1.067258	NS	NS
CD40	0.8104344	NS	NS
CD83	0.8622541	NS	NS
CSF1R	−0.4019432	NS	NS
CSF3	0.2317648	NS	NS
GH	0.2433543	NS	NS
IFI27	1.0259721	NS	NS
IFI35	0.8819949	0.5279851	0.5328957
IFIH1	2.0319667	1.2292278	1.2140828
IL10	0.2751558	NS	NS
IL18	1.5629684	NS	NS
IL23A	0.2042142	NS	NS
IRF9	0.3081433	0.3495631	NS
ISG20	1.7618855	0.7512296	0.963982
MSH2	−0.5418109	NS	NS
MYD88	−0.1989387	NS	NS
PARP9	0.5359386	0.5820352	NS
PSMB8	0.3486514	NS	NS
PSMB9	1.7753032	0.8993551	0.9559519
PSME2	0.7497557	NS	NS
TAP1	1.853143	1.0263434	1.0151106
TAP2	0.8709287	NS	NS
TAPBP	0.3379066	NS	NS
TLR3	0.7228488	NS	NS
TNF	2.3677108	NS	NS

The table shows the log₂(fold change) in mRNA expression calculated from the microarray data for a number of innate immune-related genes at 8 h after infection with the indicated virus. NS = no significant change.

determined by LIMMA, were examined by functional module analysis based on KEGG pathways, by comparison with canonical pathways, by analysis of associated Gene Ontology (GO terms) and by construction of networks of related genes (Ingenuity Pathway Analysis (IPA)). All of these methods revealed the same biological processes occurring in the infected cells. At 2 hpi, MV-infected cells showed upregulation of a number of type-1 interferon-regulated genes (e.g. IFIT3 and IFIT5) and other genes involved in innate immune responses (CCL2, XAF1 and RGS16), as well as proteins that regulate other transcriptional pathways (TRIM25 and CDKN2AIP). The activation of innate immune responses was even more obvious at 4 hpi, with upregulation of a number of interferon-responsive and other innate immune response genes, as illustrated in the IPA-generated network in Fig. 3. In contrast, RPV-infected cells did not show statistically significant upregulation of any individual gene until 8 hpi. The degree of activation of IFN-stimulated and innate immune-related genes was lower in RPV-infected cells at 8 hpi than that seen in MV-infected cells at 4 hpi (Fig. 3); responses to RPV-S were even lower than to RPV-R (Table 1).

These observations suggested that MV infection caused a rapid induction of the type 1 interferon (IFN) response, while this response was much delayed in RPV infection. Since neither IFN- α nor IFN- β was represented on either array, we carried out real-time PCR assay of bovine IFN- β mRNA in the samples to confirm these observations (Fig. 4). We found that IFN- β mRNA levels were already increased in MV-infected cells relative to mock-infected cells at 2 hpi, rose to a peak at 8 hpi and then declined, while the levels in RPV-infected cells increased only at later times and never reached the same level as seen in the MV-infected cells. As expected from the microarray analysis, interferon mRNA levels were slightly higher in RPV-R-infected cells than in RPV-S-infected cells.

In addition to the interferon response, MV-infected cells showed a strong tumour necrosis factor (TNF) response, with TNF transcripts being clearly upregulated at 8 hpi (Table 1, Fig. S2). The TNF response may start earlier than this, as Gene Set Enrichment Analysis (GSEA) showed similarities between the transcriptome response to MV infection at 2 hpi and 4 hpi and that elicited by treatment of cells with TNF, indicating some TNF secretion even at early time points. MV-infected cells at 8 hpi showed increased transcription of a number of apoptosis-related genes (e.g. caspases 1 and 8 (CASP1 and CASP8), tumour necrosis factor (ligand) superfamily member 10 (TNFSF10/TRAIL), CD40 and various proteasome components) (Fig. 5), which may be a response to the increased expression of TNF. However, as moDCs functionally model many of the characteristics of primary immature DCs, the increased expression of CD40 is more likely to be a reflection of cellular activation/maturation, while

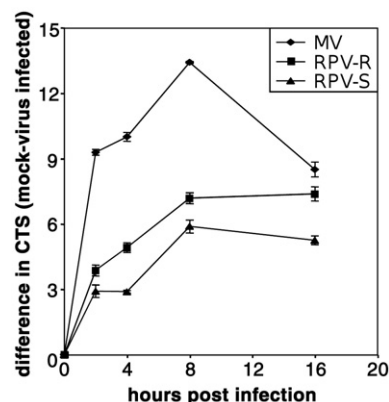
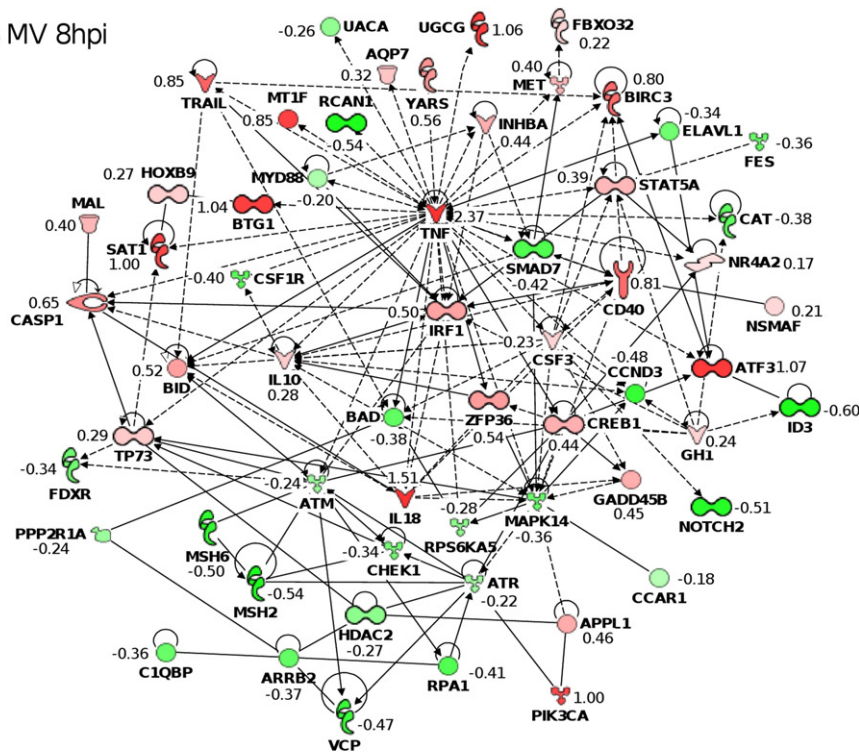


Fig. 4. Quantification of IFN- β mRNA in infected cells. Real-time PCR determination of bovine IFN- β mRNA was performed on total RNA from virus-infected and mock-infected cells as described in Materials and methods. The amount of mRNA in virus-infected samples relative to that in mock-infected cells is expressed as the difference in Ct values. Error bars represent the standard error of the difference in mean values (mock minus virus-infected).

(a) MV 8hpi



(b) RPV-R 16hpi

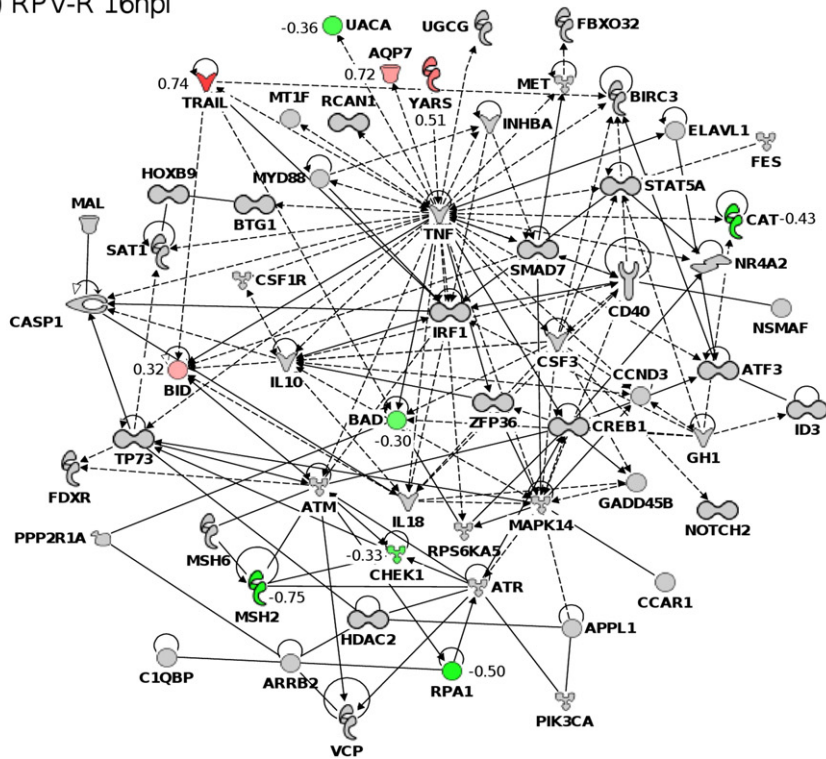


Fig. 5. Effect of MV infection on apoptotic genes. (a) The list of apoptosis-related genes showing altered expression in MV-infected cells at 8 hpi when compared to mock-infected cells at the same time was used to construct a network of all genes/gene products having known interactions. The estimated \log_2 (fold change in expression level) relative to mock-infected cells is shown for each gene with colouring to indicate upregulated (red) or downregulated (green) transcripts. (b) The same network overlaid with the data for RPV-R-infected cells at 16 hpi when compared to mock-infected cells at the same time. In RPV-S-infected cells at 16 hpi when compared to mock-infected cells at the same time, only TRAIL showed significant change (upregulated). Legend as Fig. 3.

the expression of CASP1 along with TNF and IL18 suggests the development of an inflammatory response, and possibly the formation of activated inflammasomes. This inflammatory response would lead to a block of apoptosis, and we did observe upregulation of the apoptosis inhibitor baculoviral IAP repeat-containing 3 (BIRC3)

as well as decreased transcription of a number of genes promoting apoptosis (e.g. BAD, CAT and NOTCH2) (Fig. 5). Interestingly, RPV-infected cells showed only limited effects on apoptosis-related genes even at the latest timepoint used (16 hpi) (Fig. 5), despite the clear induction of interferon in these cells. MV-infected cells also showed

early downregulation of components of the mitogen activated protein kinase (MAPK) cascade and, by 8 hpi, downregulation of transcripts for RNA polymerase I and II components and proteins involved in cell cycle control (e.g. CCND3 (cyclin D3), CDK4 (cyclin-dependent kinase 4), MAPK14, SEPT4 (septin 4) and CDC23 (cell division cycle 23 homologue)), observations not made on cells infected with RPV.

Direct comparison of MV infection with RPV infection showed primarily the expected differences arising from the different cytokine responses, in particular the difference in type 1 IFN induction. Relative to RPV infection, MV-infected cells also showed downregulation of genes from a number of metabolic pathways (e.g. glutathione metabolism, fatty acid biosynthesis and glycolysis) and upregulation of genes of the inositol phosphate metabolism pathway. Comparison of the responses to infection by vaccine or virulent RPV was more difficult due to the smaller number of genes showing individual significant changes in transcription level. GSEA, in which patterns of change in multiple genes are analysed (Subramanian et al., 2005), is more useful in such cases. Comparison of RPV-R and RPV-S using GSEA showed, in addition to the effects of the slightly higher IFN levels in RPV-R infection, that RPV-R infection induced a relative upregulation of cell cycle regulator proteins, and downregulation of genes controlling cell proliferation. Genes expressed at lower levels in RPV-R infected cells, relative to RPV-S-infected cells, at 8 and 16 hpi resembled the pattern of downregulation induced by overexpression of p21/CDKN1a (cyclin-dependent kinase inhibitor 1A), suggesting that RPV-R sends infected cells into p53-mediated G1 arrest. Interestingly, this pattern was not seen in MV-infected cells.

The observed differential cytokine induction would have been expected to have a significant effect on viral replication. Since the length of the timecourse was insufficient to allow new virions to accumulate, we measured viral RNA transcription, specifically the levels of viral nucleocapsid (N) protein mRNA, as an indicator of viral replication. Real-time PCR of RPV and MV N mRNA levels showed that all three mRNAs were already present in quite high amounts at 2 hpi for all viruses (Fig. 6). The effect of the induction of innate immune responses by MV could be seen by the fact that MV N mRNA levels failed to increase further over the following 14 h, whereas N mRNA levels for both strains of RPV show a second increase at around 8 hpi, representing either transcription from newly synthesised genome, a process that takes at least 3 h even under optimal conditions and with plentiful supply of viral proteins (Gubbay, Curran, and Kolakofsky, 2001) or increased transcription arising due to *de novo* synthesised viral polymerase (Plumet et al., 2005).

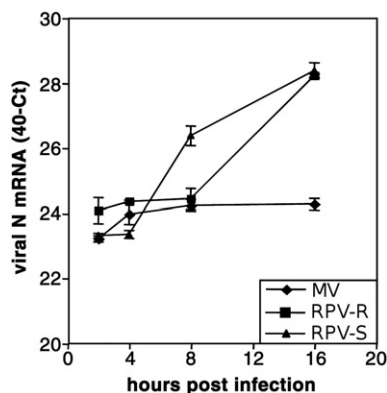


Fig. 6. Quantification of viral mRNA. Nucleocapsid protein mRNA for RPV-S, RPV-R or MV was determined by real-time RT-PCR using specific primer pairs as described in Materials and methods and expressed as the difference between the observed Ct and 40 (total number of cycles used) in order to make increases in mRNA levels lead to an increase in graphed value. Error bars show one standard deviation of the measured values.

Discussion

DCs are immune cells unique in their ability to initiate host innate and adaptive immune responses. They are distributed throughout the tissues of the host, forming a continuous network of sentinels detecting invading pathogens. They are likely to be one of the first cells encountered by either RPV or MV, and certainly the first type of cell encountered that carries the RPV/MV receptor CD150. Due to DCs' critical role in the immune response, many viruses have evolved complex mechanisms for subverting or altering DC functions in order to promote the pathogen's survival. The direct interactions of viruses with DCs are fundamental to understanding viral pathogenicity and its relationship to host responses. The growth of MV in various types of DC and the roles of DCs in MV pathogenicity and virus spread has been studied by several groups (recently reviewed in Hahm (2009)). Transcriptome analyses permit examination of the host response at the level of a specific cell type; in the particular case of DCs, useful inferences can be made about the nature of the ensuing immune response in the host itself.

Comparison of the response of the bovine moDCs to infection with virulent and avirulent RPV suggests that one of the main differences is in the strength of the type 1 IFN response. This finding agrees with studies in both animals and primary cell culture which suggested that virulent RPV induced a weaker IFN response than attenuated virus strains (el-Zein and Sour, 1985; Hussain et al., 1982) and also with our more recent findings in cell culture which showed that the non-structural C protein of RPV-S is more effective than that of the vaccine strain RPV-R at inhibiting IFN induction, and in consequence that RPV-R activates transcription from the IFN- β promoter more than does RPV-S (Boxer et al., 2009). The ability of a strain of RPV to block the induction of interferon, whether in primary cells or cell lines, is not an absolute guide to virulence, however, since the wild-type ancestor of the RPV-R vaccine strain causes essentially 100% mortality in cattle yet induces significant amounts of interferon in cell culture (Boxer et al., 2009). This may be because the overall type 1 IFN response in the host is largely governed by IFN production in plasmacytoid DCs (pDCs), and so the responses of conventional DCs, or any of the other cell types used in these and related studies, are only a limited guide to the overall innate immune response in the host and hence the outcome of infection. While there is as yet no marker for bovine pDCs allowing their purification, it would be of interest to see a comparison of the effects of MV infection on transcription in human pDCs with the results from the existing studies (Zilliox et al., 2006; Sato et al., 2008).

The response of the bovine moDCs to MV infection throws up several interesting questions. At first glance it seems as if the replication of MV in cattle is blocked purely because the virus induces a strong innate immune response in bovine cells. However, a previous study on the effect of wild-type MV on transcription in human monocytes showed an almost identical rapid induction of IFN- β (Zilliox et al., 2006), showing that the effect of wild-type MV on bovine and human cells is similar. A separate study with another wild-type MV isolate showed the same rapid innate immune response in the human embryonic kidney cell line 293 (modified to express the MV receptor CD150/SLAM) (Sato et al., 2008). In the latter study, a cell line derived from human cord blood (COBLa) did not show the same stimulation of the innate immune response when infected with MV; however, the ability of those cells to produce interferon in response to cytoplasmic dsRNA or other viral RNA was not shown, and they may represent a cell type unresponsive to these stimuli. Other studies suggest that the cell type may also be critical in determining the type 1 IFN response to MV. In a study using peripheral blood lymphocytes specifically free of monocyte-lineage (adherent) cells (Naniche et al., 2000), wild-type MV induced less IFN than vaccine strains, and suppressed IFN production induced by secondary infection with an MV vaccine strain.

In all three cases where microarrays have been used to assess responses to MV infection in human cells, the virus stocks were passed in cell culture several times before use, if only to create a virus stock for use in the infection studies. It may well be that even this limited passage alters the virus. The response to wild-type MV infection seen by us and others was similar to what might have been expected if the virus stocks had a high content of DIs. As described in **Materials and methods**, we took particular care to prevent DIs from accumulating, and the almost constant virus titre at each passage suggested no significant accumulation of DIs. We also looked for growth suppression interference by our MV stock in a mixing experiment (Whistler et al., 1996). MV DI would be expected to interfere equally with MV or with RPV since MV promoters are replicated as well as RPV promoters by RPV proteins (Baron and Barrett, 1997; Brown et al., 2005). When we mixed the MV stock with RPV-S stock (which clearly did not have a significant DI content, given the minimal response to RPV-S infection seen in the moDCs), no reduction in virus yield was seen (data not shown). Similarly, the other studies which showed strong interferon responses to wild-type MV (Sato et al., 2008; Zilliox et al., 2006) took pains to prevent DI accumulation in their virus stocks.

The data from our studies comparing RPV and MV, combined with the other studies on MV alone, indicate a fundamental difference between RPV and MV. The second major observation in these studies was the greatly delayed response to RPV infection in the DCs. DCs are functionally adapted to mount an immediate response to infection whether by viral or bacterial pathogen. The rapid transcriptional response induced by MV is what might normally be expected. The observation that the transcriptional responses in RPV-infected cells did not reach detectable levels until >4 hpi suggests that RPV is blocking/inhibiting such responses in an active way, and that MV does not have the same blocking mechanism. Having established that it is the non-structural proteins of RPV that separately control interferon action and induction (Boxer et al., 2009; Nanda and Baron, 2006), we are also studying the ability of the non-structural proteins of the MV isolate used in these studies to affect interferon action and/or induction, since the response of the moDCs to infection by these viruses suggests that there are real differences in the way these proteins work. The RPV C protein blocks IFN induction at a point downstream of interferon regulatory factor 3 (IRF3) activation (Boxer et al., 2009), and can therefore block the induction of IFN whether it occurs via the cytoplasmic pathogen recognition receptors such as retinoid inducible gene 1 (RIG-I) or via extracytoplasmic receptors such as Toll-like receptors (TLRs) (Boxer, E.L. and Baron, M.D., unpublished). Since RPV C appears to interfere with the activity of both IRF3 and NF- κ B (Boxer et al., 2009), it may be inhibiting a range of transcriptional responses in addition to the effect on IFN induction. The C protein of the MV used in these studies blocks IFN induction induced by cytoplasmic dsRNA, but not that which is induced via TLRs (Boxer, E.L. and Baron, M.D., unpublished), so the mechanisms involved appear to be fundamentally different. RPV has evolved mechanisms to suppress IFN induction by a number of different pathways, while MV seems to have adopted a strategy that allows it to replicate, in humans at least, in the face of a strong type 1 IFN response in at least some cell types (Sato et al., 2008; Zilliox et al., 2006). Such a difference in strategy would have implications for the risk of MV crossing into animal populations, or the risk of animal morbilliviruses crossing into human populations if MV is eradicated and vaccination stopped. If MV and RPV used the same strategy to cope with their hosts' innate immune responses, the barrier to humans being pathogenically infected by RPV or PPRV might only be the need for a mutation in one viral protein to allow it to fit better with the same target protein in the new host, a relatively low barrier for an RNA virus. If the viruses have had to evolve different strategies to cope with (possibly subtle) differences in their hosts' different innate immune

systems, then there is a much higher barrier preventing one virus from causing disease in another host.

Further work is needed to determine the mechanism of stimulation of the innate immune responses in the case of MV. The intracellular response to MV infection has been ascribed to melanoma differentiation associated protein-5 (mda-5) (Berghall et al., 2006) or to RIG-I (Plumet et al., 2007); wild-type MV has also been reported as specifically activating the TLR2-dependent signalling pathway (Bieback et al., 2002), which leads to an inflammatory response as seen here. Inflammatory responses have been seen in other cell types infected with wild-type MV and localised inflammatory responses are thought to be associated with the encephalitis associated with some MV infections (Ghali and Schneider-Schaulies, 1998; Patterson et al., 2003), although MV seems not to generate a systemic inflammatory response (Devaux et al., 2008). It is clearly important to determine the effects of these viruses on the full range of immune effector cell types, particularly on pDCs.

Materials and methods

Virus growth and purification

The virus strains used in this study were (a) the Saudi/81 strain of RPV, which causes 100% mortality in cattle (RPV-S), used at the 4th passage from a sample of spleen from an animal infected with RPV-S and used as an example of a wild-type, highly pathogenic strain of this virus; (b) the "Plowright" vaccine strain of RPV, which is an apathogenic, non-secreted strain derived by tissue culture passage (RPV-R) and used at the 4th passage after recovery from a full-length cDNA (Baron and Barrett, 1997); and (c) an isolate of MV from the 2000 outbreak of measles in Dublin, derived from a tissue sample of a patient with severe measles (a kind gift from Dr P. Duprex, Queen's University, Belfast), used at the 9th passage after receipt as the original isolate was heavily contaminated with mycoplasma and was therefore grown through three rounds of treatment (6 passages) with BM-cyclin (Merk) before a stock was grown for use. The titre of the virus stock was measured at each passage and remained between 10^5 and 10^6 , indicating that the MV stock did not have significant contamination with defective interfering particles (DIs) (Whistler, Bellini, and Rota, 1996). All working stocks of virus were grown in the marmoset B-cell line B95a, which has previously been shown to be a good host for wild-type morbilliviruses (Kobune et al., 1991) and to prevent accumulation of DIs (Shingai et al., 2007). Virus was purified from soluble factors that might contribute to the host cell response by precipitation with polyethylene glycol (PEG) (Trepanier et al., 1981). Final virus titre was determined as 50% tissue culture infectious dose (TCID₅₀) on B95a cells. As a further control, mock-infected B95a cells were processed as for virus purification and the resultant PEG precipitate used to 'infect' cells ("Mock" samples).

Preparation and infection of moDCs

Monocyte-derived dendritic cells (moDCs) were prepared as described (Werling et al., 1999) from fresh blood from Holstein-Friesian stock animals. Cells (approx 10^5 per well in 12-well plates) were infected with virus at a multiplicity of infection (m.o.i.) of 10 for 1 h in a final volume of 0.5 ml. Virus was then removed, cells washed once with medium and incubated for 2, 4, 8 or 16 h before harvesting. Cells were lysed and RNA prepared using the RNeasy mini kit (Qiagen) as described by the manufacturer. Purified RNA was further treated with Turbo DNA-free (Ambion) to remove any residual cell DNA. RNA quality was checked by analysing all samples on an Agilent Bioanalyser using the RNA 6000 Nano kit, with no samples showing detectable degradation or DNA contamination. RNA concentration was measured by Nanodrop.

Amplification and labelling of RNA

1 µg total RNA was amplified with simultaneous incorporation of amino-allyl UTP using the Amino Allyl MessageAmp™ II aRNA amplification kit (Ambion) according to the manufacturer's protocol. The quality of the amplified RNA (aRNA) was checked using the Agilent Bioanalyser and quantified using the Nanodrop. aRNA was labelled with AlexaFluor 555 or 647 using the succinyl-ester derivatives (Invitrogen). Each vial of dye was resuspended in 11 µl DMSO. aRNA (10 µg) was dried and redissolved in 9 µl coupling buffer (Ambion), added to the dissolved dye and incubated for 30 min at room temperature. The reaction was quenched by adding 4.5 µl 4 M hydroxylamine and incubating for a further 15 min. The labelled aRNA was then purified using the filter cartridges provided with the Message Amp II kit, eluting with 30 µl H₂O. Dye incorporation was determined spectrophotometrically using the Nanodrop, and was 1 dye per 20–30 bases.

Microarrays and hybridisation

The arrays used in these studies were the bovine long oligo (BLO) array and the bovine total leukocyte (BOTL5) array, both obtained from the Center for Farm Animal Genomics, University of Michigan. The BLO array consists of approximately 8400 70mers representing predicted bovine mRNAs spotted in duplicate, plus a number of bovine control sequences ("housekeeping" genes) spotted multiple times, as well as negative control spots. The BOTL5 array has cDNAs representing 1391 bovine genes selected from a bovine leukocyte library (Coussens and Nobis, 2002), all spotted in duplicate, with other spots representing negative controls and bovine housekeeping genes.

Hybridisation was performed in ArrayHyb#3 buffer (Ambion) using a Hybstation 4800 (Tecan). Each labelled aRNA was divided into two; half was used to hybridise to a BLO array and half to a BOTL array. All aRNAs were cohybridised with other experimental samples in an interwoven loop design (incomplete block design in which each array is a block) (Fig. S1 in Supplemental Materials). Samples from the six experimental animals were randomly allocated to each 'block' (cohybridisation) to avoid bias. The resultant two-channel data from the arrays were normalised using the routines in the LIMMA package. All raw microarray data and protocols have been deposited in the Array Express database (<http://www.ebi.ac.uk/microarray-as/ae/>) with accession number E-MEXP-1856.

Data analysis

Arrays were scanned on a GenePix 4000B scanner. Segmentation was performed with SpotFinder (Niles Scientific Inc) for BLO arrays or with BlueFuse (BlueGnome) for BOTL arrays. The array data was normalised (Smyth and Speed, 2003) using the 'R' package LIMMA; control spots were eliminated and duplicate spots averaged before further analysis. LIMMA was used to fit a linear model to the data in which dye, animal and treatment (virus/time) were factors. From the initial linear model, contrasts were extracted comparing virus-infected with mock-infected at each time or each virus with other viruses at each time. These contrasts were used to determine the array features showing differential expression (Smyth, 2004), which were taken as features where the LIMMA coefficient (fitted value of log₂ (fold change in transcription)) was statistically different from 0 at $p < 0.05$ after adjustment for multiple comparisons. The results of both sets of contrasts (virus vs mock and virus vs virus) for the BLO arrays are provided in Supplemental Materials as a Microsoft Excel file (BLO array data.xls).

Data from the LIMMA analysis was used for Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Normalised two-colour data were converted back to single-colour data for principal

component analysis (PCA) of the data. PCA was performed using the `prcomp()` function in 'R'. Gene Set Expression Analysis was carried out on the single-colour data using the package GSEA from the Broad Institute (Mootha et al., 2003; Subramanian et al., 2005) after mapping the bovine genes represented by each array feature to the corresponding human homologue where this was known. Functional module analysis (Marston et al., 2009; Ortega et al., 2008; Sameith et al., 2008) was performed by summarizing the activity of a gene subset associated with a given KEGG pathway by computing the first two principal components (PCs). In order to identify PCs which were associated with differences in the responses of cells infected with different viruses we applied the SAM statistical test (Tusher et al., 2001).

Real-time quantitative RT-PCR

For quantification of mRNA transcripts, primer pairs were selected using Primer3 (Rozen and Skaletsky, 2000) based on the relevant bovine mRNA sequence. RPV N mRNA was quantified using a primer pair matching a highly conserved region of the N mRNA and designed so that the primer annealing sites are identical between the RPV-S and RPV-R strains. The MV N mRNA was quantified using the MV multi-strain primers described in Hummel et al. (2006) and the results confirmed using the N mRNA primer pair described by El Mubarak et al. (2005). Optimal annealing temperatures were determined for each primer pair and the primers checked for absence of primer dimers before use. The primers used and the optimum annealing temperature for PCR for each pair are listed in Table I in Supplemental Materials. Equal amounts of aRNA (80 µg) for each treatment-time combination were pooled across animals; reverse transcription was carried out on 120 ng of the pooled RNA using anchored oligo(dT) ((dT)₁₆VN) as the primer and Superscript III (Invitrogen) essentially as described by the manufacturer. The resultant cDNAs were diluted four-fold and heated at 70 °C for 15 min. PCR was carried out using JumpStart Sybr Green mix (Sigma); each reaction contained 2 µl diluted cDNA and 2 pmol each of the forward and reverse primers. Duplicate PCRs were carried out from each of duplicate cDNAs. As all the commonly used housekeeping genes we have examined show some level of change in response to RPV infection, we relied on accurate measurement of RNA concentration to ensure comparability of samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.virol.2009.09.031](https://doi.org/10.1016/j.virol.2009.09.031).

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